Acute lung injury: apoptosis in effector and target cells of the upper and lower airway compartment

Roth Z'graggen, B ; Tornic, J ; Müller-Edenborn, B ; Reyes, L ; Booy, C ; Beck-Schimmer, B

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Acute lung injury: Apoptosis in effector and target cells of the upper and lower airway compartment

Birgit Roth Z'graggen [$], Jure Tornic [#$], Björn Müller-Edenborn[#$], Livia Reyes [$], Christa Booy [$], and Beatrice Beck-Schimmer [#$]

[$] Institute of Physiology and Centre for Integrative Human Physiology, University of Zurich, Zurich, Switzerland

[#] Institute of Anesthesiology, University of Zurich, Zurich, Switzerland

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Corresponding author:
Beatrice Beck-Schimmer, M.D.
Institute of Physiology and Centre for Integrative Human Physiology
Institute of Anaesthesiology
University of Zurich Medical School
Winterthurerstrasse 190
CH-8057 Zurich
Switzerland
Phone: +41 44 635 50 35, Fax: +41 44 635 68 14
E-mail: **Beatrice_Beck.Schimmer@access.uzh.ch**

ABSTRACT

Apoptotic cell death has been considered an underlying mechanism in acute lung injury. To evaluate the evidence of this process, apoptosis rate was determined in effector cells (alveolar macrophages, neutrophils) and target cells (tracheobronchial and alveolar epithelial cells) of the respiratory compartment upon exposure to hypoxia and endotoxin stimulation *in vitro*. Cells were exposed to 5% oxygen or incubated with lipopolysaccharide (LPS) for 4, 8 and 24 h, and activity of caspase-3, -8, and -9 was determined. Caspase-3 of alveolar macrophages was increased at all three time points upon LPS stimulation, while hypoxia did not affect apoptosis rate at early time points. In neutrophils, apoptosis was decreased in an early phase of hypoxia at 4 h. However, enhanced expression of caspase-3 activity was seen at 8 and 24 h. In the presence of LPS a decreased apoptosis rate was observed at 8 h compared to controls, while it was increased at 24 h. Tracheobronchial as well as alveolar epithelial cells experienced an enhanced caspase-3 activity upon LPS stimulation with no change of apoptosis rate under hypoxia. While increased apoptosis rate is triggered through an intrinsic and extrinsic pathway in alveolar macrophages, intrinsic signalling is acitvated in tracheobronchial epithelial cells. The exact pathway pattern in neutrophils and alveolar epithelial cells could not be determined. These data clearly demonstrate that upon injury each cell type experiences its own apoptosis pattern. Further experiments have to be performed to determine the functional role of these apoptotic processes in acute lung injury.

INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) cause severe respiratory failure and death in critically ill patients. The development of ALI/ARDS is associated with several clinical disorders including direct pulmonary injury from pneumonia and aspiration as well as indirect pulmonary injury from trauma or sepsis [1]. Although knowledge about mechanisms leading to ALI/ARDS has increased, no specific and successful treatment options exist to date and thus the mortality rate remains high in patients with ALI/ARDS [2].

The airway compartment with alveolar macrophages and epithelial cells such as tracheobronchial and alveolar epithelial cells is a physiological barrier to a variety of environmental agents including gases, particulates and microbes. Alveolar macrophages are located at the air-tissue interface in the lung and are therefore the first cells, which interact with inhaled organisms and antigens [3]. It has been shown in several lung injury models that activated pulmonary macrophages and stimulated epithelial cells release cytokines and chemokines [4, 5]. All these inflammatory mediators together play a crucial role in the orchestration of an inflammatory response, particularly in neutrophil recruitment, representing a different type of effector cells. Neutrophil sequestration and migration into alveoli remain pathohistological hallmarks of ARDS, with neutrophils being key effector cells, which further destruct lung tissue [6].

The process of programmed cell death, or apoptosis is known to play a major regulatory role in maintaining many biologic processes, not least of which is the inflammatory response, such as in ALI/ARDS. Two major apoptosis pathways in mammalian cells are known so far: 1) the intrinsic or mitochondrial pathway with involvement of Bcl-2 at the outer membrane of mitochondria, cytochrome c release,

and activation of caspase-9 2) extrinsic or death receptor pathway with activation of caspase-8 upon binding of death activator to Fas- and tumor necrosis factor (TNF)-receptor at the surface of the cell. Both pathways converge at the level of caspase-3 activation [7]. Apoptosis results in destruction of proteins by caspases as well as in fragmentation of the DNA. Finally, apoptotic cells are eliminated by phagocytes. Inappropriate activation or inhibition of apoptosis can lead to disease either because ‘undesired’ cells develop prolonged survival or because ‘desired’ cells die prematurely [8].

The purpose of this study was to evaluate *in vitro* apoptosis rate and pathway of effector and target cells at different time points upon injury with endotoxin and hypoxia, both factors, with might contribute to ALI *in vivo*. We were interested to assess if upon injury different cell types undergo apoptosis in a similar way. Our hypothesis was that within the group of effector or target cells, the cells would experience the same kind of apoptosis.

MATERIAL AND METHODS

Animals

Specific pathogen-free male Wistar rats (250-300 g) were purchased from Janvier (Le Genest-St. Isle, France). Rats were anesthetized with subcutaneously administered Narketan (Ketamin 10%, Kepro, Utrecht, Holland) 0.8 - 1 ml/kg and Rompun (Xylazin 2%, Streuli Pharma, Uznach, Switzerland) 0.25 - 0.5 ml/kg. All animal experiments and animal care were approved by the Swiss Veterinary Health Authorities.

Alveolar macrophages

Alveolar macrophages (CRL-2192, American Type Culture Collection, Rockville, MD) were established from normal Sprague-Dawley rat alveolar macrophage cells obtained by lung lavage, cloned and subcloned three times. The cells exhibit characteristics of macrophages and are sensitive to endotoxin. Cells from passages not higher than 5 were used. Cells were cultured in nutrient mixture F-12 Ham (Ham's F-12; Invitrogen Corporation, Carlsbad, CA), completed with 15% fetal bovine serum (FBS), 5% penicillin/streptomycin (10'000 U/l) and 5% HEPES. Overnight, before starting the experiments, cells were incubated with Ham's F-12 with 1% FBS.

Isolation of neutrophils

Human neutrophils (polymorphonuclear cells, PMN) were isolated by gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Dubendorf, Switzerland) followed by 1% dextran sedimentation for 1 h to separate neutrophils from erythrocytes, as described previously [9]**.** After centrifugation of the supernatant, contaminating erythrocytes were lysed with distilled water followed by the addition of

2.7% NaCl to stop hypotonic lysis. Neutrophils were washed with phosphate-buffered saline (PBS) and resuspended at a total concentration of 2 x 10^6 PMN/ml in DMEM/1% FBS.

Isolation and culture of tracheobronchial epithelial cells

Trachea and bronchial parts of the respiratory system were excised, ligated at the distal ends, filled with 0.01% protease type XIV (Sigma, Buchs, Switzerland) and incubated overnight at 4°C [10]. Tracheobronchial epithelial cells were flushed out with FBS, washed twice, and incubated in airway epithelial cell basal medium (PromoCell, Heidelberg, Germany)/10% premium FBS (BioWhittaker, Verviers, Belgium)/1% penicillin/streptomycin in 96 well plates, previously coated with 50 μg/ml rat tail collagen (Sigma, Buchs, Switzerland) for 30 min at room temperature. Cells reached 100% confluency within 3 days. Purity was verified using periodic acid-Schiff staining (>98%). Epithelial cell character was also independently confirmed by a pathologist at the University Hospital of Zurich, performing cytokeratin staining.

Alveolar epithelial cells

L2 cells (CCL 149, American Type Culture Collection, Rockville, MD) are isolated cell lines derived through cloning of adult female rat lung of alveolar epithelial cell type II origin [11]. Cells from passages 4 - 12 were used. The cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Invitrogen AG, Basel, Switzerland), supplemented with 10% FBS), 1% penicillin-streptomycin, and 1% HEPES buffer and grown in uncoated 96 well plates (Corning Inc., Corning, NY) to more than 95% confluence. Prior to cell stimulation, the medium was changed to DMEM/1%FBS.

Hypoxia

A cell incubator (Bioblock, Ittigen, Switzerland) adjustable to different oxygen concentrations by insufflation of nitrogen (N_2) was used as a hypoxic cell chamber. The concentrations were monitored continuously by an oxygen sensor. Experiments were performed with 5% oxygen and 5% CO_2 at 37°C. For control cells, an incubator (Bioblock) with 21% O_2, 5% CO_2 at 37°C was used. For our studies, all four cell types were plated in 96-well tissue culture plates (Corning, New York, NY) and exposed to 5% O_2 for 4, 8 and 24 h.

Stimulation with lipopolysaccharide (LPS)

Cells were washed twice and incubated with lipopolysaccharide from *Escherichia coli* serotype 055:B5 (LPS; 20 µg/mL; Sigma-Aldrich, Buchs, Switzerland) (or PBS as a control) for 4, 8 and 24 h at 37°C.

Fluorometric assays for caspase -3,-8 and -9 activity

For the caspase assays, alveolar macrophages, neutrophils, tracheobronchial and alveolar epithelial cells were stimulated with LPS (20 µg/ml) or camptothecin as positive control (4 µM), or exposed to hypoxia for 4, 8 and 24 h. Caspase-3 activity was determined by measuring proteolytic cleavage of the fluorogenic caspase-3 substrate Ac-Asp-Glu-Val-Asp-AMC, the fluorogenic caspase-8 substrate Z-Ile-Glu-Thr-Asp-AFC (Calbiochem, Läufelfingen, Switzerland) and the fluorogenic caspase-9 substrate Ac-Leu-Glu-His-Asp-AFC (Merck, Darmstadt, Germany). Cells were incubated for 1 h at 37°C with 125 µM substrate. The fluorescence of the cleaved reporter group was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. Camptothecin (an extract of the Chinese tree Camptotheca

acuminata) is a potent inhibitor of topoisomerase I, a molecule required for DNA synthesis. Camptothecin has been shown to induce apoptosis and was therefore used for positive controls.

Flow cytometric measurement of apoptosis

The percentage of apoptotic and nectrotic cells was quantified by performing a cell staining with annexin V and 7-amino-actinomycin D (7-AAD) (PE Annexin V Apoptosis Detection Kit I, BD Biosciences, NJ). Apoptosis was directly quantified with annexin V, measuring the translocation of phospholipids phosphatidylserines from the inner to the outer leaflet of the plasma membrane in apoptotic cells. The loss of membrane integrity in late apoptotic or necrotic cells was assessed by 7-AAD staining. 7-AAD intercalates into double-stranded nucleic acids. It is excluded by viable cells, but can penetrate cell membranes of dying or dead cells. For analysis, a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NY) was used.

Statistical analysis

Results are expressed as median and the error bars are plotted as median with interquartile range for the caspases assays. Values from stimulated cells are shown as % compared to control values of 100 %. All experiments were conducted at least 4 times. ANOVA and Kruskal-Wallis multiple comparison tests were performed to assess the statistical significance of differences, using GraphPad Prism 4.0 software (San Diego, CA). For flow cytometry analysis, boxplots were designed using the SPSS program. P values < 0.05 were considered significant.

RESULTS

Hypoxia- and endotoxin-induced apoptosis in effector cells

To determine a possible effect of hypoxia on apoptosis in alveolar macrophages and neutrophils, caspase-3 as the key enzyme in the final pathway was determined as well as caspase-8 and -9 to distinguish between intrinsic and extrinsic pathways. Interestingly, the two cell types, although belonging to the group of effector cells, did not experience the same changes. While under hypoxic conditions apoptosis rate did not change in alveolar macrophages at early time points compared to control cells, caspase-3 activity increased by 80% in the LPS group and caspase-8 activity showed a 3-fold increase in the same group after 4 h ($p < 0.05$) (**Fig. 1A**). After 8 h, caspase-3 activity was enhanced by 240%, caspase-8 activity by 148% and caspase-9 activity by 85% in the LPS group ($p < 0.05$) (**Fig. 1B**). **Fig. 1C** shows the 24 h caspase-3, -8 and -9 activities with no significant changes, except again for the LPS group, where caspase-3 level was increased by 277%, caspase-8 level by 41%, and caspase-9 by 198% ($p < 0.05$).

Neutrophils, however, showed less caspase-3 activity under hypoxia in comparison to control cells after 4 h (29% decrease, $p < 0.05$) (**Fig. 2A**), while caspase-3 activity was significantly higher after 8 and 24 h (**Fig. 2B, C**). With LPS, neutrophils experienced a decrease in caspase-3 and caspase-8 activity at 8 h ($p < 0.05$) (**Fig. B**), while a five-fold increase of caspase-3 was observed at 24 h compared to control cells ($p < 0.05$) (**Fig. 2C**).

Hypoxia- and endotoxin-induced apoptosis in target cells

Hypoxia did not alter apoptosis rate in tracheobronchial epithelial cells within 24 h of exposure to 5% oxygen (**Fig. 3A-C**), while stimulation with LPS increased caspase-3

activity by 129% and caspase-9 activity by 80% at 4 h of incubation ($p < 0.05$) (**Figure 3A**). After 8 h of LPS stimulation, a 79 % increase of caspase-3 activity was observed, while caspase-9 was 2 fold higher compared to the control group ($p < 0.05$) (**Fig. 3B**). At 24 h, caspase-3 activity reached 206% and caspase-9 95% compared to the adequate control group with 100% expression ($p < 0.05$) (**Fig. 3C**).

Alveolar epithelial cells as possible target cells showed a different apoptosis pattern as tracheobronchial epithelial cells. Hypoxia did not induce changes in apoptosis rate in alveolar epithelial cells, while LPS increased caspase-3 activity by 56%, 78% and 70% after 4, 8 and 24 h, respectively, (all p values < 0.05) (**Figure 4A-C**). No changes of caspase-8 and -9 activity were observed upon LPS injury for all time points (**Fig. 4A-C**).

As the increase of caspase activities might not necessarily correlate with the process of apoptosis, neutrophils were analyzed assessing apoptosis-induced cellular changes. Flow cytometric measurements of annexin V staining showed that changes of caspases reflect the process of apoptosis (Fig. 5A, 5B). At 4 hours of injury, apoptosis rate decreased by 19% (range 35%) under hypoxia and by 32% (range 39%) with LPS, repectively ($p < 0.05$). In tracheobronchial epithelial cells, apoptosis increased upon 24 h of LPS stimulation as previously shown with the help of a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining (10).

DISCUSSION

Numerous studies have been conducted to better understand ALI/ARDS. Cell death has been demonstrated to play a key role in the lung during the pathogenesis of ALI/ARDS. In this study we focused on different cell types of the respiratory compartment and determined apoptosis *in vitro* in the model of hypoxia- or endotoxin-induced injury. Alveolar macrophages, tracheobronchial cell as well as alveolar epithelial cells showed a similar apoptosis response pattern to injuries such as hypoxia or LPS: 1) No increased apoptosis rate was observed under hypoxia at early time points. 2) For all three cell types, LPS induced apoptosis at any time point. In alveolar macrophages, LPS stimulation activated caspase-3, caspase-8 and caspase-9, while in tracheobronchial epithelial expression of caspase-9 and caspase-3 was increased. In alveolar epithelial cells, only caspase-3 seemed to be enhanced upon LPS stimulation. Neutrophils, however, reacted differently with a caspase-3 decrease at 4 h and a subsequent increase at 8 and 24 h under hypoxic conditions. LPS also induced an attenuation of the apoptosis rate at 8 h of stimulation, with an increase of caspase-3 at 24 h. In both cell types - neutrophils and alveolar epithelial cells - the type of apoptosis pathway (internal/external) could not be identified, while activation of apoptosis in alveolar macrophages was triggered by the internal and external pathway, in tracheobronchial epithelial cells by the internal one.

Programmed cell death is a process by which cells ‘commit suicide’ through apoptosis or other alternative pathways. Cell death occurs at a specific point in the developmental process and is therefore, considered as ‘programmed’. It can also be triggered by external stimuli, such as soluble cell death ligands, which are released during inflammatory responses, or intrinsic stimuli, resulting from alteration of cellular function and metabolism. Apoptosis is characterized by cell shrinkage and formation

of apoptotic bodies. Various biochemical features of apoptosis have been identified, which have been used frequently as an indication for apoptosis, such as caspase activation, DNA fragmentation, and externalization of phosphatidylserine, a cell surface marker for phagocytosis [7]. Caspases are the most exensively studied proteases that are activated during apoptosis. They exist as inactive protease precursors within cells and can be activated by themselves or by other proteases. The intrinsic or mitochondrial pathway is triggered by Bcl-2 at the outer membrane of the mitochondria, leading to cytochrome c release. Cytochrome c binds then to the apoptotic protease activating receptor-1 (Apaf-1). This Apaf-1/cytochrome c complex allows the interaction of procaspase-9 with Apaf-1, thus placing pro-caspase-9 molecules in close proximity with each other and promoting their activation [12]. The extrinsic pathway of apoptosis is initiated upon ligation of death activators such as TNF, Fas ligand and TNF-related apoptosis-inducing ligand to the cell surface death receptors. Activated death receptors recruit and activate multiple pro-caspase-8 molecules with activation of caspase-8 [13]. Both an intrinsic and extrinsic pathway end up with the activation of caspase-3.

LPS has been commonly used and also recommended as a tool to study the mechanisms of ALI in cultured cells and in animals [6]. In a model of intratracheal LPS administration in hamsters, extended apoptosis was observed in alveolar epithelial cells after 24 h of injury [14]. Also another study, performed *in vitro* in primary culture of rat alveolar type II cells, underlines the result that increased apoptosis rate is observed upon stimulation with LPS after 48 h [15]. Additionally, MacRedmond et al. obtained similar apoptosis results in an *in vitro* study in human alveolar epithelial cells and a 24-h-stimulation of LPS [16]. Our results support these findings with increased apoptosis rate in alveolar epithelial cells upon an extended time of stimulation with LPS. We further point out, that apoptosis is also observed in

the early phase of endotoxin stimulation. Therefore, apoptosis seems to be present independent of the time of LPS stimulation. This statement can also be applied to tracheobronchial epithelial cells. In a previous work form our group, we were able to demonstrate that the intrinsic apoptosis pathway is activated at 24 h of LPS stimulation [10]. Results of the current study show that the process of apoptosis is initiated already at earlier time points upon stimulation with LPS. In accordance with epithelial cells alveolar macrophages experience the same process of apoptosis with increased activity of caspase-3 in acute and subacute situations of LPS exposure. Another study underlining these findings was performed by Bingisser et al. [17]. This group showed that only LPS induced apoptosis rate of human alveolar macrophages, but not cytokines.

An important aspect of apoptosis in epithelial cells of the respiratory compartment and in alveolar macrophages is the cellular signalling pathway. While tracheobronchial epithelial cells undergo apoptosis over the intrinsic pathway, the intrinsic and extrinsic signalling is activated in alveolar macrophages. For alveolar epithelial cells, the pathway is not clear as both caspases-8 and -9, respectively, are not involved. Further experiments have to be performed to determine the exact pathway in these cells. A possible explanation might be the modification of the cell line compared to primary culture of alveolar epithelial cells.

Interestingly, while no change in caspase-3 activity of neutrophils was detected at 4 h of LPS stimulation, it significantly decreased at 8 h. At the time point of subacute injury at 24 h, however, a five-fold increase of apoptosis rate was detected. These results are in accordance with previous studies. Upon stimulation with various concentrations of LPS (1 – 100 ng/ml), apoptosis rate decreased concentration dependently after 12 h of stimulation [18]. Also Hirata et al. found a depressed apoptosis rate in neutrophils upon LPS stimulation [19]. A study

performed in patients with severe sepsis showed that spontaneous neutrophil apoptosis seemed to be inhibited in these patients compared to healthy volunteers [20]. Keel et al. isolated neutrophils from healthy humans and patients with severe sepsis and stimulated them with LPS for 16 h, showing a decrease in apoptosis rate in neutrophils from healthy persons, while apoptosis did not change upon stimulation in neutrophils from septic patients. In a model of ALI, induced by intravascular injection of oleic acid to simulate pulmonary fat embolism-induced ALI, at 1 and 4 h following oleic acid injection a massive neutrophil response was found in the lung, without any evidence of apoptosis [21]. 24 h later, in the early resolution stage, intense neutrophil apoptosis was observed. Therefore, it might be concluded that neutrophils experience a different apoptosis response over time and in comparison with other cell types of the respiratory compartment at an early time point of injury. In a first phase of acute injury, a delay in apoptosis would provide neutrophils with a longer life span, possibly inducing or aggravating injury as described in patients with sepsis and sepsis-induced ARDS [22]. In a later phase about resolution of an injury, apoptosis rate increases.

Under hypoxic conditions, apoptosis rate of epithelial cells and alveolar macrophages did not change. Neutrophils, however, experienced again a different reaction regarding apoptosis rate compared to the other cell types. Hypoxia decreased caspase-3 activity in neutrophils after 4 h of exposure, while at time points of 8 and 24 h, caspase-3 activity was increased. Current data indicate that many factors operating at the inflamed site such as hypoxia and acidosis serve a dual function in both priming and activating neutrophils by delaying apoptosis as well as decreased accumulation and function by increasing apoptosis [23]. As observed for alveolar epithelial cells, activation pathway of apoptosis is not clear in neutrophils.

In conclusion, our data show that the three cell types from the respiratory compartment alveolar and trachebronchial epithelial cells as well as alveolar macrophages show the same pattern of apoptosis regarding caspase-3 activity upon exposure to endotoxin and hypoxia. The apoptotic answer of neutrophils, however, is different. The functional implications of these inflammatory answers have further to be analyzed.

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Fig. 1.

Determination of apoptosis rate in alveolar macrophages. Caspase-3 (3), -8 (8) and -9 (9) activity was measured in control cells (co), after exposure to 5% oxygen (hyp), or exposure to 20 μg/ml lipopolysaccharide (LPS) for 4 h (A), 8 h (B) and 24 h (C). Camptothecin was used as a positive control (pos co) for apoptosis. Values are median with interquartile range from 5 experiments. * P< 0.05 between control and injured cells.

Fig.2.

Determination of apoptosis rate in neutrophils. Caspase-3 (3), -8 (8) and -9 (9) activity was measured in control cells (co), after exposure to 5% oxygen (hyp), or exposure to 20 μg/ml lipopolysaccharide (LPS) for 4 h (A), 8 h (B) and 24 h (C). Camptothecin was used as a positive control (pos co) for apoptosis. Values are median with interquartile range from 5 experiments. * P< 0.05 between control and injured cells.

Fig. 3.

Determination of apoptosis rate in tracheobronchial epithelial cells. Caspase-3 (3), -8 (8) and -9 (9) activity was measured in control cells (co), after exposure to 5% oxygen (hyp), or exposure to 20 μg/ml lipopolysaccharide (LPS) for 4 h (A), 8 h (B) and 24 h (C). Camptothecin was used as a positive control (pos co) for apoptosis. Values are median with interquartile range from 5 experiments. * P< 0.05 between control and injured cells.

Fig. 4.

Determination of apoptosis rate in alveolar epithelial cells. Caspase-3 (3), -8 (8) and -9 (9) activity was measured in control cells (co), after exposure to 5% oxygen (hyp), or exposure to 20 μg/ml lipopolysaccharide (LPS) for 4 h (A), 8 h (B) and 24 h (C). Camptothecin was used as a positive control (pos co) for apoptosis. Values are median with interquartile range from 5 experiments. * P< 0.05 between control and injured cells.

Figure 5

Flow cytometric analysis of annexin V and 7-amino-actinomycin D (7-AAD) staining of neutrophils after a 4 h exposure to 5% oxygen, or exposure to 20 μg/ml lipopolysaccharide (LPS). Camptothecin was used as a positive control for apoptosis. A) Blot from a typical example (4 experiments were performed). B) Results from 4 experiments summarized as boxplot. * P< 0.05 between control and injured cells.